Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Feline Calicivirus in Cell Culture

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Supplemental Assay Method for Titration of Feline Calicivirus in Cell Culture

1. Introduction

This is an *in vitro* titration method for assaying modified-live feline calicivirus (FCV) vaccines for viral content. The method uses plaque forming units (PFU) in a cell culture system for titration of FCV.

2. Materials

2.1 Equipment/instrumentation

2.1.1 2-ml self-refilling repetitive syringe[1]

2.1.2 Micropipettor: 200 µl[2]

2.1.3 Blender[3]

2.1.4 1000-ml borosilicate glass media bottle with screw-top lid[4]

2.1.5 36° ± 2°C, 5% ± 1% CO_2, high humidity incubator[5] meeting the requirements of the current version of GDOCSOP004

2.1.6 Water bath[6]

2.1.7 Vortex mixer[7]

2.2 Reagents/supplies

2.2.1 FCV Reference Virus[8]

2.2.2 Crandell feline kidney[9] (CRFK) cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

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[1] Wheaton 13-689-50C, Fisher Scientific, Inc., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

[2] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

[3] Waring blender, Cat. No. 14-509-35, Fisher Scientific, Inc. or equivalent

[4] Wheaton 219760, Fisher Scientific, Inc. or equivalent

[5] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

[6] Cat. No. 15-461-10, Fisher Scientific, Inc. or equivalent

[7] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent

[8] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[9] CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

2.2.3 4-well tissue culture plates[10]

2.2.4 Minimum Essential Medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts without bicarbonate[11]

2.2.4.2 2.2 g sodium bicarbonate ($NaHCO_3$)[12]

2.2.4.3 Q.S. to 1000 ml with deionized water (DW), and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[13]

2.2.4.4 Sterilize through 0.22-µm filter.[14]

2.2.4.5 Aseptically add:

1. 100 units/ml penicillin[15]
2. 50 µg/ml gentamicin sulfate[16]
3. 100 µg/ml streptomycin[17]
4. 5 ml lactalbumin hydrolysate or edamin[18]

2.2.4.6 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 900 ml of MEM

2.2.5.2 Aseptically add:

1. 100 ml fetal bovine serum (FBS), heat inactivated at 56° ± 2°C for 30 ± 5 min

2. 10 ml L-glutamine[19]

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[10]Cat. No. 7603705, ICN Biochemicals, Inc., 3300 Hyland Ave., Costa Mesa, CA 92626 or equivalent

[11]Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[12]Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[13]Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[14]Disposable filter, Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

[15]Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

[16]Gentocin solution, Cat. No. 0061-0464-04, Schering Laboratories or equivalent

[17]Cat. No. S-9137, Sigma Chemical Co. or equivalent

[18]Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[19]L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

2.2.5.3 Store at 4° ± 2°C.

2.2.6 2X Medium

2.2.6.1 100 ml 10X MEM[20]

2.2.6.2 2.6 g $NaHCO_3$

2.2.6.3 340 ml DW

2.2.6.4 Sterilize through 0.22-µm filter.

2.2.6.5 Aseptically add:

1. 5 ml lactalbumin hydrolysate or edamin
2. 100 units/ml penicillin
3. 50 µg/ml gentamicin sulfate
4. 100 µg/ml streptomycin
5. 50 ml FBS, heat-inactivated

2.2.6.6 Store at 4° ± 2°C.

2.2.7 2% Tragacanth Gum (Trag)

2.2.7.1 20 g Trag[21]

2.2.7.2 1000 ml DW

2.2.7.3 Mix vigorously, small amounts at a time, with a blender set on high.

2.2.7.4 Pour 500 ml each into 1000-ml media bottles.

2.2.7.5 Sterilize by autoclaving at 15 psi for 35 ± 5 min.

2.2.7.6 Store at 4° ± 2°C.

2.2.8 Overlay Medium

2.2.8.1 Mix equal volumes of 2X Medium and 2% Trag.

[20]Cat. No. 11435, Life Technologies, Inc. or equivalent
[21]Acros AC42138-5000, Fisher Scientific, Inc. or equivalent

2.2.8.2 Store at 4° ± 2°C.

2.2.9 70% Ethyl Alcohol

2.2.9.1 73 ml ethyl alcohol[22]

2.2.9.2 27 ml DW

2.2.9.3 Store at room temperature (RT) (23° ± 2°C).

2.2.10 Crystal Violet Stain

2.2.10.1 7.5 g crystal violet[23]

2.2.10.2 50 ml 70% Ethyl Alcohol

2.2.10.3 250 ml formaldehyde[24]

2.2.10.4 Dissolve crystal violet in alcohol, add remaining ingredients.

2.2.10.5 Q.S. to 1000 ml with DW.

2.2.10.6 Filter through filter paper.[25]

2.2.10.7 Store at RT.

2.2.11 12 x 75-mm polystyrene tubes[26]

2.2.12 25-ml pipette[27]

2.2.13 3-ml syringe[28] and 20-ga x 1½-in needle[29]

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[22]Denatured, 190 proof, Cat. No. 7018, J.T. Baker, Inc. or equivalent
[23]Cat. No. C 0775, Sigma Chemical Co. or equivalent
[24]37% by weight, Cat. No. F79, Fisher Scientific, Inc. or equivalent
[25]Whatman #1, Cat. No. 1001, Fisher Scientific, Inc. or equivalent
[26]Falcon 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent
[27]Cat. No. 13-675-30, Fisher Scientific, Inc. or equivalent
[28]Leur-Lok®, Cat. No. 309585, Becton Dickinson Labware or equivalent
[29]Cat. No. 250107, Becton Dickinson Labware or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses.

3.2 Preparation of equipment/instrumentation

3.2.1 Set the water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK Plates

3.3.1.1 Multiple 4-well plates are seeded with CRFK cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 6 hr of incubation at 36° ± 2°C in a CO_2 incubator. Cells older than 72 hr cannot be used in the test. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent 48 hr after seeding.

3.3.2 Preparation of Reference Virus

3.3.2.1 Rapidly thaw a vial of FCV Reference Virus in a 36° ± 2°C water bath.

3.3.2.2 Dispense 1.8 ml of MEM into each of 8, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-8} using a 2-ml repetitive syringe.

3.3.2.3 Transfer 200 µl of the Reference Virus to the tube labeled 10^{-1}, mix with the vortex mixer. Discard pipette tip.

3.3.2.4 Transfer 200 µl from the 10^{-1} labeled tube to the 10^{-2} tube, mix with the vortex mixer. Discard pipette tip.

3.3.2.5 Repeat Step **3.3.2.4** for each of the subsequent dilutions transferring 200 µl from the previous tube to the next dilution tube.

3.3.3 Preparation of Trag

3.3.3.1 Warm the Trag in 36° ± 2°C water bath for 60 ± 10 min prior to Step **4.6**. Approximately 35 ml per plate is required.

3.4 Preparation of the sample

3.4.1 Rehydrate a vial of the Test Vaccine according to the manufacturer's instructions using a syringe and needle. Allow to incubate for 15 ± 5 min at RT.

3.4.2 Dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6} using a 2-ml repetitive syringe.

3.4.3 Transfer 200 µl from the Test Vaccine to the tube labeled 10^{-1}, mix with the vortex mixer. Discard pipette tip.

3.4.4 Transfer 200 µl of the tube labeled 10^{-1} to the tube labeled 10^{-2}, mix with the vortex mixer. Discard pipette tip.

3.4.5 Repeat Step **3.4.4** for each subsequent dilution through 10^{-6} transferring 200 µl from the previous tube to the next dilution tube.

4. Performance of the test

4.1 Decant the Growth Medium from the CRFK Plates.

4.2 Inoculate 1 well/dilution with 200 µl/well from dilutions 10^{-6} through 10^{-3} of the Test Vaccine into CRFK Plates. **Note:** The same pipette tip may be used if starting with the most dilute dilution. Gently rotate the plates to evenly disperse the inoculum.

4.3 Inoculate 1 well/dilution with 200 µl/well of the dilutions 10^{-8} through 10^{-6} of the Reference Virus. Gently rotate the plates to evenly disperse the inoculum.

4.4 One uninoculated well serves as a negative cell control.

4.5 Incubate the inoculated CRFK Plates at 36° ± 2°C for 75 ± 15 min in a CO_2 incubator.

4.6 Add 8 ml/well of the Overlay Medium (see section **2.2.8**) to the plates with a 25-ml pipette. Discard any unused, warmed Overlay Medium.

4.7 Incubate the CRFK Plates undisturbed at 36° ± 2°C for 96 ± 12 hr in a CO_2 incubator.

4.8 At the end of incubation, without removing the Overlay Medium, pipette 5 ml of the Crystal Violet Stain (see section **2.2.11**) into each well of the plates with a 25-ml pipette.

4.9 Allow plates to incubate at RT 25 ± 5 min.

4.10 Wash the Overlay Medium and the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container with running water from the cold water tap. Allow to air dry.

4.11 PFU counting. If feline rhinotracheitis virus (FRV) and FCV plaques are counted together in a combination vaccine, the FRV plaques will contrast markedly from FCV. The FRV plaques are small, clear, and approximately 1 mm in diameter, with a distinct edge.

4.11.1 The FCV PFU are visible as large, clear, circular areas (averaging 3 mm to 4 mm in diameter) with fuzzy edges in the cell monolayer where the cells have been destroyed by the virus.

4.11.2 Count the number of FCV PFU for each well.

4.12 The titer is expressed as PFU per dose of vaccine and is calculated as follows, using the plaque count from the dilution well which contains 10-100 PFU:

Example:

Log_{10} of PFU (65)	= 1.8
Log_{10} of reciprocal of dilution counted (10^{-3})	= 3.0
Log_{10} of reciprocal of dose factor $\frac{200\ \mu l\ inoculum}{1\text{-ml dose}} = \frac{1}{5}$	= 0.7
Total	= 5.5

Titer of the vaccine is $10^{5.5}$ PFU per ml dose.

5. Interpretation of the test results

5.1 For a valid assay, the calculated titer of the FCV Reference Virus must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.2 The uninoculated cell controls cannot exhibit any plaques, cytopathic effects, or cloudy media that would indicate any contamination.

5.3 For a satisfactory result, the titer of the Test Vaccine must be equal to or greater than the titer specified in the Outline of Production.

5.4 If the titer is less than the titer specified in the Outline of Production, then the Test Vaccine may be retested as stated in 9 CFR 113.8(b)(2).

6. Report of test results

6.1 Report test results as PFU/dose.

6.2 Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.314.

7.2 Cottral GE: 1978, Manual of standardized methods for veterinary microbiology. Comstock Publishing Associates, p. 731. Ithaca, NY.

8. Changes

8.1 This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.